PESQUISA VETERINÁRIA BRASILEIRA
Brazilian Journal of Veterinary Research
ISSN 0100-736X (Print)
ISSN 1678-5150 (Online)

Pesq. Vet. Bras. 44:e07366, 2024
DOI: 10.1590/1678-5150-PVB-7366

Original Article
Small Animal Diseases

Occurrence of pathogens transmitted by *Rhipicephalus sanguineus sensu lato* ticks in dogs in the semiarid region of Rio Grande do Norte state, Brazil[1]

Larissa L.C. Nogueira[2], Juliana F.V. Braga[3]*, Raylanne L.P. Sousa[2], Bruno V.S. Araújo[2], Ana L.C.G. Guimarães[2], Larissa D.A.O. Carmo[2], Wesley A.C. Coelho[4] and Sthenia S.A. Amora[2]

ABSTRACT.- Nogueira L.L.C., Braga J.F.V., Sousa R.L.P., Araújo B.V.S., Guimarães A.L.C.G., Carmo L.D.A.O., Coelho W.A.C. & Amora S.S.A. 2024. **Occurrence of pathogens transmitted by *Rhipicephalus sanguineus sensu lato* ticks in dogs in the semiarid region of Rio Grande do Norte state, Brazil**. *Pesquisa Veterinária Brasileira 44:e07366, 2024*. Departamento de Ciência Animal, Universidade Federal Rural do Semi-Árido, Rua Francisco Mota 572, Presidente Costa e Silva, Mossoró, RN 59625-900, Brazil. E-mail: juliana.braga@ufpi.edu.br

The aim of this study was to determine the occurrence of infection by *Ehrlichia canis*, *Anaplasma platys*, *Babesia vogeli*, and *Hepatozoon canis* in dogs from the semiarid region of Rio Grande do Norte state. Also, we evaluated the characteristics that favor the infection by *E. canis* and the presence of *Rhipicephalus sanguineus s.l.* ticks. For that, 120 dogs were included, from which blood samples were collected for DNA extraction and molecular diagnosis of these four pathogens. Anamnesis and physical examination were performed on each patient, and all properties were characterized. Chi-square and Fisher's exact tests were used to verify the association of the studied variables. The most prevalent pathogen in the study was *E. canis* (13.3%), followed by *A. platys* (11.7%), *H. canis* (6%) and *B. vogeli* (6%). Correspondence analysis performed between *E. canis* positivity and the variables studied showed the influence of factors such as tick history, non-vaccination and non-use of antiparasitic medications. The main environmental factor observed in the infection by *E. canis* was the presence of trees and vegetation in the residences. Recognizing these characteristics can help elaborate prevention and control strategies since environmental management activities seek to reduce the interaction between vector and host and, consequently, the exposure to diseases.

INDEX TERMS: Ticks, *Rhipicephalus sanguineus*, environmental management, vector-borne diseases, PCR, semiarid region.

RESUMO.- [**Ocorrência de patógenos transmitidos pelo carrapato *Rhipicephalus sanguineus sensu lato* em cães na região semiárida do estado do Rio Grande do Norte, Brasil**]. O objetivo deste estudo foi determinar a ocorrência de infecção por *Ehrlichia canis*, *Anaplasma platys*, *Babesia vogeli* e *Hepatozoon canis* em cães da região semiárida do estado do Rio Grande do Norte. Ainda, foram avaliadas as características que favorecem a infecção por *E. canis* e a presença de carrapatos *Rhipicephalus sanguineus s.l.* Para tanto, foram incluídos 120 cães, dos quais foram coletadas amostras de sangue para extração de DNA e diagnóstico molecular desses quatro patógenos. Foi realizada anamnese e exame físico de cada paciente e todas as propriedades foram caracterizadas. Os testes Qui-quadrado e exato de Fisher foram utilizados para verificar a associação das diferentes variáveis estudadas. O patógeno de maior prevalência no estudo foi *E. canis* (13,3%), seguido por *A. platys* (11,7%), *H. canis* (6%) e *B. vogeli* (6%). A análise de correspondência realizada entre a positividade de *E. canis* e as variáveis estudadas, demonstrou influência de fatores como histórico de carrapatos, ausência de vacinação e ausência de uso de medicações antiparasitárias. Os principais fatores ambientais observados na infecção por *E. canis* foram a presença de árvores e jardins nas residências.

[1] Received on March 12, 2024.
Accepted for publication on April 9, 2024.
[2] Universidade Federal Rural do Semi-Árido (UFERSA), Rua Francisco Mota 572, Presidente Costa e Silva, Mossoró, RN 59625-900, Brazil.
[3] Universidade Federal do Piauí (UFPI), BR-135 Km 3, Planalto Horizonte, Bom Jesus, PI 64900-000, Brazil. *Corresponding author: juliana.braga@ufpi.edu.br
[4] Faculdade de Enfermagem e Medicina Nova Esperança, Av. Presidente Dutra 701, Alto de São Manoel, Mossoró, RN 59628-000, Brazil.

O reconhecimento dessas características pode auxiliar na elaboração de estratégias de prevenção e controle, visto que atividades de manejo ambiental buscam reduzir a interação entre vetor e hospedeiro e, consequentemente, a exposição a doenças.

TERMOS DE INDEXAÇÃO: Carrapatos, *Rhipicephalus sanguineus*, manejo ambiental, doenças transmitidas por vetores, PCR, região semiárida.

INTRODUCTION

The *Rhipicephalus sanguineus sensu lato* (*s.l.*) tick, also known as the brown dog tick, has a cosmopolitan distribution, is strongly associated with its canine host, and is one of the main vectors of pathogens of medical and veterinary importance (Dantas-Torres 2010, Dantas-Torres & Otranto 2014, Tucker et al. 2021). Factors such as urbanization, increased travel and commercial exchanges, climate change, and ecological changes influence the geographic dispersion and population expansion of these ticks (Wikel 2018), which contributes to an increase in the number of cases of diseases transmitted by these arachnids (Lindahl & Grace 2015, Barradas et al. 2020).

Owing to their nesting habits and endophilic behavior, *R. sanguineus s.l.* ticks usually inhabit burrows and artificial shelters. This vector is predominantly found in urban and suburban areas where it lives in close proximity to dogs and humans. In these areas, particularly among dogs that inhabit confined environments and are not treated with ectoparasiticides, the degree of infestation is usually high (Lorusso et al. 2010, Gray et al. 2013).

In addition to parasitism, ticks can transmit important infections in their hosts, representing a considerable diagnostic challenge, as they usually cause non-specific signs. Their clinical manifestations can vary according to the pathogen involved, parasitemia level, and the dog's immunological status (Baneth 2011, Rojas et al. 2014). The brown dog tick highlighted here is the main vector of a large number of pathogens such as *Babesia vogeli*, *Ehrlichia canis*, *Hepatozoon canis*, and *Anaplasma platys*. Dogs are usually infected with *B. vogeli*, *E. canis*, and *A. platys* through the bite of infected ticks, whereas *H. canis* infection occurs through the ingestion of *R. sanguineus s.l.* (Rucksaken et al. 2019). Although brown dog ticks have been extensively studied, there is a lack of information regarding the environmental characteristics that may favor their appearance and, consequently, the transmission of diseases.

This study aimed to determine the occurrence of infection by *E. canis*, *A. platys*, *B. vogeli*, and *H. canis* in dogs from the semiarid region of Rio Grande do Norte state, Brazil, and to evaluate the characteristics that favor the *E. canis* infection and the presence of *R. sanguineus s.l.* ticks. Identifying these factors and their correlation with the presence of the diseases will contribute to developing environmental management measures that control the proliferation of the vector and, consequently, its contact with humans.

MATERIALS AND METHODS

"Ethical approval. The protocol was approved by the Ethics Committee on the Use of Animals (CEUA) of the "Universidade Federal Rural do Semi-Árido" (UFERSA), number 03/2019.

Study area, dogs, and samples. The study was carried out in the municipality of Mossoró, Rio Grande do Norte (05°11'16.8'' S, 37°20'38.4'' W). The city has a hot semiarid climate with a rainy season in the summer, delaying until autumn. It has an average temperature of 27.4°C, irregular annual rainfall, and an average relative humidity of 68.9%.

In this study, 120 dogs were included, regardless of sex, breed, and age, and they were treated at a private veterinary clinic.

This descriptive cross-sectional study and the sample size calculation for infinite populations followed recommendations proposed by Heinrich et al. (2021) through the formula $n=Z^2_{\propto/2}.P(1-P)/e^2$. Considering a confidence level (Z) of 1.96 and a success probability (P) of 50%, which provides a maximum sample size and a margin of error (*e*) of 9%, the total sample size amounted to 119 dogs.

Four milliliters of blood were collected from the dogs included in the study in a tube containing anticoagulant (EDTA) by jugular venipuncture for DNA extraction and molecular diagnosis.

Ethical issues and epidemiological data. During patient care, tutors were informed about the research objectives and instructed to read the Free and Informed Consent Form. After acceptance and signature, anamnesis and a physical examination of the dog were performed. At this stage, ocular mucous membranes were evaluated to identify anemia or jaundice. In addition, other symptoms such as body temperature, presence of ectoparasites, signs of apathy, anorexia, weight loss, and dehydration were investigated. Clinical alterations were registered in individual clinical-epidemiological records.

At the time of anamnesis, the tutors were asked about the accessibility of the animal to the street, vaccination and coexistence with other animals, history of infestation with ectoparasites, and other questions that could arise according to the individuality of each patient.

The visit to the properties where the included animals reside was carried out to identify the environmental characteristics of those areas by searching for factors that favor the presence of *Rhipicephalus sanguineus s.l.* and, consequently, greater exposure of animals to the transmitter of diseases.

Characterization of properties. The properties of all the animals participating in the study were visited. The visits to the homes were carried out by one of the project members, with the tutors' authorizations via signed Free and Informed Consent Forms.

Residences were characterized by the presence of environmental characteristics that favor the appearance of the vector, such as the presence of other domestic animals, trees, or decaying wood; the presence of vegetation in the peridomicile or green area close to the residence; cracks in the walls; accumulation of construction; and presence of nearby water courses.

DNA extraction and PCR. For DNA extraction, the PureLink® Genomic DNA Mini Kit (Thermo Fisher Scientific) was used according to the manufacturer's instructions. After extraction, the quality and concentration of the DNA were analyzed using a spectrophotometer (NanoDrop™ Lite, Thermo Scientific), and the samples were stored at -20°C until PCR. The extracted DNA was subjected to PCR to detect the endogenous control gene (dog beta-actin) and the study agents (*Anaplasma platys, Babesia vogeli, Ehrlichia canis*, and *Hepatozoon canis*).

Oligonucleotide primers and specific amplification programs previously published and detailed in Table 1 were used for DNA detection. For the PCRs, 200 to 300ng of DNA from the samples and 12.5pmol of each primer were used in a final reaction volume of 25µL (PCR Supermix Brasil, Invitrogen®). Nested PCR was used to amplify *E. canis* DNA, using 1µL of the product from the first PCR as a sample for the second reaction. Amplification of *A. platys*, *B. vogeli*, and *H. canis* DNA was performed by conventional PCR. For all reactions, DNA from samples known to be positive for the investigated agents was used as a positive control, and diethyl pyrocarbonate (DEPC) water was used as a negative control.

The amplified products were subjected to 1.5% agarose gel electrophoresis in 1x Tris-Acetate-EDTA (TAE) buffer, with SybrSafe® as a DNA evidencing dye, for 60 min at 100 volts. A 100bp molecular weight marker (Promega®) was used to determine the size of the amplified products, following the manufacturer's instructions. After electrophoresis, the gel was visualized in an ultraviolet light transilluminator (ProteinSimple®) and recorded using the AlphaImager Mini system program.

The samples were considered positive for the dog beta-actin, *B. canis* 18S rRNA, *E. canis* 16S rRNA, *H. canis* 18S rRNA, and *A. platys* 16S rRNA genes when amplified products with an approximate size of 98bp (Turchetti 2014), 602bp (Duarte et al. 2008), 389bp (Bulla et al. 2004), 306bp (Kaur et al. 2020) and 359bp (Mathew et al. 1997) respectively, were detected.

Statistical analysis. Data are expressed as simple frequency and percentage values obtained through the Statistical Package for the Social Sciences (SPSS) version 23.0 statistical program. The chi-square and Fisher's exact tests were used to verify the association of the different variables studied in relation to the studied diseases. The latter was used when the expected frequency was less than five. In this sense, odds ratios (OR) with respective 95% confidence intervals (95% CI) were calculated. Finally, correspondence analysis was performed to determine the relationship between variables related to canine ehrlichiosis. The level of significance established was 5%.

RESULTS

The prevalence of the pathogens investigated in this study was 13.3% (16/120) for *Ehrlichia canis*, 11.7% (14/120) for *Anaplasma platys*, 5% (06/120) for *Hepatozoon canis*, and 5% (06/120) for *Babesia vogeli*. Seven animals presented co-infections with these pathogens; one dog was simultaneously positive for *E. canis, H. canis*, and *B. vogeli*, four dogs were

Table 1. Oligonucleotide sequence, amplification programs and size of PCR amplified products for dog beta-actin (endogenous control), *Babesia vogeli, Ehrlichia canis, Hepatozoon canis* and *Anaplasma platys* used in this research

Target gene	Primer	Oligonucleotide sequence (5'-3')	Amplification program	Products (pb)	Reference
Dog beta-actin	Actb-F	GGCATCCTGACCCTGAAGTA	Initial denaturation: 95°C, 10' 35 cycles of: Denaturation: 95°, 30'' Girdling: 60°C, 30'' Extension: 72°C, 30'' Final extension: 72°C, 5'	98pb	Turchetti (2014)
	Actb-R	CGCAGCTCGTTGTAGAAGGT			
B. vogeli (18S rRNA)	BAB1	GTGAACCTTATCACTTAAAGG	Initial denaturation: 94°C, 2' 35 cycles of: Denaturation: 94°, 30'' Girdling: 56°C, 30'' Extension: 72°C, 60'' Final extension: 72°C, 5'	602pb	Duarte et al. (2008)
	BAB4	CAACTCCTCCACGCAATCG			
E. canis (16S rRNA)	1st reaction: EHO-F	AGAACGAACGCTGGCGGCAAGCC	Initial denaturation: 94°C, 10' 40 cycles of: Denaturation: 94°, 60'' Girdling: 60°C, 60'' Extension: 72°C, 60'' Final extension: 72°C, 4'	478pb	Bulla et al. (2004)
	1st reaction: EHO-R	CGTATTACCGCGGCTGCTGGC			
	2nd reaction: ECA-F	CAATTATTTATAGCCTCTGGCTATAGGAA	Initial denaturation: 94°C 10' 35 cycles of: Denaturation: 94°, 60'' Girdling: 60°C, 60'' Extension: 72°C, 60'' Final extension: 72°C 4'	89pb	
	2nd reaction: ECA-R	TATAGGTACCGTCATTATCTTCCCTAT			
H. canis (18S rRNA)	HC-18S-F	CACCAGGTCCAGACATAGAAAG	Initial denaturation: 95°C 3' 35 cycles of: Denaturation: 95°, 30'' Girdling: 62°C, 30'' Extension: 72°C, 45'' Final extension: 72°C, 1'	306pb	Kaur et al. (2020)
	HC-18S-R	AAGCTTACCAGCCAAGGTTAT			
A. platys (16S rRNA)	EPLAT5-F	TTTGTCGTAGCTTGCTATGAT	Initial denaturation: 95°C 5' 35 cycles of: Denaturation: 94°, 30'' Girdling: 58°C, 30'' Extension: 72°C, 45'' Final extension: 72°C 5'	359pb	Mathew et al. (1997), Matei et al. (2016)
	EPLAT3-R	CTTCTGTGGGTACCGTC			

simultaneously positive for *H. canis* and *B. vogeli*, and two dogs were simultaneously positive for *E. canis* and *A. platys*.

Clinical signs found during physical examination were anorexia, weight loss, polydipsia, claudication, onychogryphosis, pale mucous membranes, fever, splenomegaly, lymphadenomegaly, corneal ulcers, and digestive and dermatological changes. Only 14 animals showed some type of clinical change during physical examination, while the other 106 were healthy.

As shown in Table 2, it was possible to observe the prevalence of positive animals for *E. canis, A. platys, B. vogeli*, and *H. canis* according to the data collected in the anamnesis. Of the dogs reactive to *E. canis*, 81.3% had access to the street, a number greater than that found for other pathogens. In addition, 56.3% of those positive for ehrlichiosis lived with other animals. Regarding the use of antiparasitic drugs, it was observed that 78.6% of dogs infected with *A. platys* used these drugs.

In Table 3, it is possible to notice the prevalence of these parasites in relation to the environmental characteristics of the animals participating in the study. Most of the positive dogs lived in houses, with the greatest proportion being 81.3% for *E. canis*-positive dogs. Among the animals positive for *A. platys*, the variable that stood out the most was the presence of vegetation, such as pasture, grass, and gardens.

Because *E. canis* was the most prevalent pathogen in the study and because of the small sample size, the correlation between disease positivity, clinical history, and environmental characteristics was determined only for this agent. It was observed that dogs that had access to the street and lived with other animals (OR, 0.98; p=0.971) or those who had a history of ticks and did not use ectoparasiticide medications (OR, 0.58; p=0.478) did not present a statistically (p<0.05) greater probability of being positive for *E. canis* in this study. However, when vaccinated and unvaccinated animals were compared, a higher prevalence of ehrlichiosis was observed in the invacilante group (p<0.03), although there was no specific vaccine for this disease.

Positivity for *E. canis* did not undergo statistical interference when the presence of other domestic animals and the type of environment the animal lived in, whether house (OR 0.73; p= 0.707) or apartment, were evaluated. Vegetation, such as pasture, shrubs, or grass, as well as green areas near the residences, did not show statistical significance. There was also no correlation between the positivity of the disease and other characteristics that could favor the appearance of *R. sanguineus s.l.*, such as kennels, cracks in walls, stones, or the absence of pest control in the environment.

In correspondence analysis, it was observed that a history of ticks and non-use of medications against these vectors seems to be more prevalent in dogs positive for *E. canis*. The only variable with statistical significance was vaccination history.

DISCUSSION

All pathogens transmitted by *Rhipicephalus sanguineus s.l.* that were analyzed were detected in the canine population in this study, with *Ehrlichia canis* (13.3%) being the most frequent

Table 2. Prevalence of animals infected by *Ehrlichia canis, Anaplasma platys, Babesia vogeli* and *Hepatozoon canis* according to the clinical history of dogs treated in the city of Mossoró/RN, 2021

Variables	*Ehrlichia canis* n=16	*Anaplasma platys* n=14	*Hepatozoon canis* n=06	*Babesia vogeli* n=06
	%	%	%	%
Street access history	81.3	57.1	66.7	66.7
Vaccination history	37.5	85.7	66.7	66.7
History of living with other animals	56.3	35.7	33.3	50.0
Ticks history	31.3	7.1	33.3	50.0
History of using antiparasitic medication	75.0	78.6	66.7	66.7

Table 3. Prevalence of animals infected by *Ehrlichia canis, Anaplasma platys, Babesia vogeli* and *Hepatozoon canis* of dogs treated according to the environmental characteristics that they were inserted. Mossoró/RN, 2021

Variables	*Erhlichia canis* n=16	*Anaplasma platys* n=14	*Hepatozoon canis* n=06	*Babesia canis* n=06
	%	%	%	%
Environment type				
House	81.3	71.4	66.7	66.7
Apartment	18.3	28.6	33.3	33.3
Tree presence	18.8	14.3	16.7	16.7
Vegetation presence				
Pasture	6.3	7.1	0.0	0.0
Grass	6.3	14.3	0.0	0.0
Bush	6.3	7.1	0.0	0.0
Garden	0.0	14.3	0.0	0.0
Green area near the house	6.3	0.0	16.7	16.7
Presence of kennel	0.0	0.0	0.0	0.0
Presence of river	0.0	0.0	0.0	0.0
Presence of wall crack	12.5	7.1	16.7	16.7
Presence of stones	12.5	14.3	16.7	16.7
Detoxed environment for less than three months	0.0	0.0	0.0	0.0

agent. Vector-borne diseases have historically been endemic to tropical regions (Dantas-Torres 2008). In some Brazilian regions, such as Goiânia/PE, the prevalence of *Ehrlichia* spp. can reach 64.7%, with infestation by *R. sanguineus s.l.* being the main risk factor (Dantas-Torres et al. 2020). In different areas of southeast Brazil, the prevalence of this disease varies from 15% to 44.7% (Macieira et al. 2005, Costa Jr. et al. 2007). In São Joaquim de Bicas/MG, Dantas-Torres et al. (2020) identified a prevalence of 37.9%. The fact that this study included animals with or without clinical signs, in addition to a limited number of samples, may have contributed to the lower number of infected animals.

The highest number of dogs positive for *E. canis*, when compared to other pathogens, was also identified in a study conducted by Rojas et al. (2014), with a prevalence of 34% for *E. canis*, 10% for *Anaplasma platys*, 8.5% for *Babesia vogeli*, and 7.5% for *Hepatozoon canis*. These data make it possible to identify the epidemiological profile of each region, providing information on which pathogens and diseases can affect animals in that location with a greater prevalence.

Co-infection between these agents is common in regions with a high level of infestation by *R. sanguineus s.l.* In a study by Sarma et al. (2019) in India, co-infections between *Babesia gibsoni, H. canis*, and *B. vogeli* were identified in 3% of the studied dog population. Rojas et al. (2014) identified co-infection between *E. canis* and *B. vogeli* (8/18), *E. canis* and *A. platys* (4/18), *E. canis* and *H. canis* (3/18), and *A. platys* and *B. vogeli* (2/18). In these studies, more than one agent was associated with more severe clinical conditions, mainly anemia, demonstrating the importance of tracking more than one pathogen in veterinary practice.

The main clinical alterations found in the dogs in the study were anemia, fever, weight loss, anorexia, splenomegaly, lymphadenomegaly, ocular lesions and gastrointestinal disorders. In addition to the diseases studied, signs such as onychogryphosis and dermatological changes may also be related to other types of diseases, such as canine visceral leishmaniasis (Queiroz et al. 2010).

In the case of anaplasmosis, the infection in female dogs was diagnosed more than in males, which may be related to the greater exposure of these female animals to the vector of the disease compared to males. Similar results were reported by Selim et al. (2021) in Egypt, with a higher prevalence of the disease in females, especially in the German Shepherd breed.

Dogs with a history of access to the street showed greater positivity for the disease when compared to those who did not go out for walks, which may be related to the greater exposure of these animals to the vector *R. sanguineus s.l.* In a study carried out by Guedes et al. (2015) in the municipality of Ituberá/BA, dogs that had contact with other dogs, whether domiciled or not, and those with a history of infestation by *R. sanguineus s.l.* were more predisposed to infection by *E. canis*.

Ectoparasiticide medications are known to prevent hemoparasitosis in dogs (Dantas-Torres 2010, Mctier et al. 2016). Even so, in this study, even dogs that used these drugs showed positive results for ehrlichiosis (75%), anaplasmosis (78.6%), hepatozoonosis (66.7%), and babesiosis (66.7%). In this case, it is important to consider that most of the products available against ticks have a direct acaricidal action and control the parasites in the animal itself. However, since 95% of these vectors are present in the environment, an effective control must also involve periodic extermination in and around the home inhabited by the dogs (Labruna & Pereira 2001, Mctier et al. 2016).

Factors such as the presence of trees, cracks in walls, and accumulation of stones were also observed in the environment frequented by positive animals. These places provide favorable conditions for the development and persistence of *R. sanguineus s.l.*, favoring its maintenance in the environment and, consequently, the spread of diseases (Dantas-Torres 2010).

It was not possible to correlate the environmental characteristics with the presence of *E. canis*. However, the most common variables in the reacting animals were the presence of trees and backyards with vegetation. The correlation of these factors with the presence of *R. sanguineus s.l.* has been studied since 1992 by Gilot et al. (1992) in France, and it has been observed that houses with gardens are more suitable biotypes for tick development than buildings. In Japan, dogs in contact with a garden two weeks before being clinically evaluated were also more likely to be infested with ticks (Shimada et al. 2003). A study by Barrantes-González et al. (2016) demonstrated that the more a dog is infested with *R. sanguineus s.l.*, the greater the possibility of seropositivity. In addition, the number of dogs per household was determined to be a risk factor for *E. canis* positivity.

Although there is no specific vaccine against ehrlichiosis, dogs vaccinated with polyvalent vaccines against viruses were less likely to acquire *E. canis* infection, as were dogs with no history of ticks and those using ectoparasiticide medications. In a study by Angelou et al. (2019), dogs that did not receive frequent antiparasitic treatment tended to be seropositive for *Ehrlichia* spp., compared to animals that received them regularly, highlighting the need to use these medications.

Although most of the environmental variables did not show statistical significance against the positivity of canine ehrlichiosis in this study, it is known that environmental intervention activities, such as periodic environmental pest control, can be carried out to reduce favorable conditions for the development of the vector *R. sanguineus s.l.* and, consequently, the diseases transmitted by this vector to dogs.

CONCLUSIONS

Ehrlichia canis was the most prevalent pathogen in this study, followed by *Anaplasma platys, Babesia vogeli*, and *Hepatozoon canis*. Co-infection with these agents was also diagnosed, demonstrating the need to track more than one pathogen in endemic regions for diseases transmitted by *Rhipicephalus sanguineus sensu lato*.

The main environmental characteristics that influenced the positivity of canine ehrlichiosis were trees and vegetation in the residences. Factors such as tick history and non-use of antiparasitic medications were also related to positivity, highlighting the need to use these medications.

Recognizing these characteristics may help elaborate prevention and control strategies based not only on dogs as individuals but also on their interaction with the environment in which they live.

Acknowledgments.- We would like to thank the "Coordenação de Aperfeiçoamento de Pessoal de Nível Superior" (CAPES) and "Universidade Federal Rural do Semi-Árido" (UFERSA) for the financial grant for research and postgraduate scholarship.

Conflict of interest statement.- The authors declare that there are no conflicts of interest.

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